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Early induction by crotoxin of biphasic frequency changes and giant miniature endplate potentials in frog muscle

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1 Following the addition of crotoxin (250 nM) at the frog neuromuscular junction, there was an initial fall in frequency of miniature endplate potentials (m.e.p.ps), followed by a secondary rise which was characterized by the appearance of large spontaneous potentials (giants, g.m.e.p.ps) and an occasional large potential of the burst type.

2 In the presence of 2-(4-phenylpiperidino)cyclohexanol (AH5183, vesicamol), an inhibitor of vesicular acetylcholine uptake, the frequency of g.m.e.p.ps induced by crotoxin was reduced.

3 The characteristic changes in m.e.p.p. frequency and amplitude distribution were absent with crotoxin in Sr-EGTA Ringer. In the presence of high concentrations of Mn (3.6 or 5.4 mM with 0.9 mM Ca), the crotoxin-induced initial fall and the onset of the secondary rise in m.e.p.p. and g.m.e.p.p. frequencies were slower. The timing of these phases was unaffected by Ca concentrations ranging from 6.3 to 0.9 mM.

4 High concentrations of Mn ions partially inhibited the phospholipase A_2 activity of crotoxin on artificial phospholipid membranes. This also supports the involvement of the Ca-dependent phospholipase A_2 subunit in both phases of the physiological action of the toxin.

5 G.m.e.p.ps were associated with a moderate increase in m.e.p.p. frequency (2–3 s^{-1}) and were of a time-course similar to that of m.e.p.ps. They persisted after washing with medium lacking Ca ions and in the presence of Ca-Mn Ringer that blocked evoked responses.

6 It is concluded that crotoxin, acting through its phospholipase A_2 subunit, produces specific disturbances of synaptic exocytosis and vesicle formation in the axolemma of the motor nerve terminal which lead to biphasic changes in m.e.p.p. frequency and the onset of large spontaneous potentials.

Introduction

Phospholipase A_2 neurotoxins that act presynaptically (see reviews by Howard & Gundersen, 1980; Lee & Ho, 1982; Strong, 1987) induce changes in miniature endplate potential (m.e.p.p.) frequency, burst activity and the onset of giant miniature endplate potentials (g.m.e.p.ps) at endplates in frog isolated muscle (Abe *et al.*, 1976; Hawgood & Santana de Sa, 1979) as well as in mammalian muscle poisoned *in vivo* (Cull-Candy *et al.*, 1976; Hawgood & Smith, 1977). At normal frog junctions, g.m.e.p.ps are rare (Fatt & Katz, 1952). Crotoxin, a member of this group of neurotoxins, has a structure consisting of two non-covalently bound subunits viz., a basic phospholipase A_2 and a biologically inactive, acidic polypeptide (Hendon & Fraenkel-Conrat, 1971; Rubsamen *et al.*, 1971) that acts as a chaperone (Bon *et al.*, 1979) for the enzymatic moiety. In this paper, we have studied (a) the effects of crotoxin on g.m.e.p.p. and m.e.p.p. frequencies at the frog motor endplate and (b) the influence of divalent cations on crotoxin's phospholipase A_2 activity in order to define the early stages of toxicity.

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Methods

Crotalus durissus cascavella venom was the gift of Professor O. Vital Brazil, Campinas, Sao Paulo, Brazil. Crotoxin was isolated from the crude venom by gel filtration on Sephadex G75, followed by ion-exchange chromatography on DEAE-cellulose (Hendon & Fraenkel-Conrat, 1971). The toxin was judged homogeneous by SDS-polyacrylamide gel electrophoresis and had the same phospholipase A_2 activity (using egg phosphatidylcholine-cholate micelles as substrate) and toxicity as previously reported by Marlas & Bon (1982) for crotoxin isolated from *C. durissus terrificus* venom.

Intracellular recordings were obtained from endplates, the majority in cutaneous-pectoris muscles but a few in sartorius muscles isolated from *Rana pipiens*, by use of conventional microelectrode techniques. M.e.p.ps were recorded both on a Mingograf 34 pen recorder and by on-line voltage sampling every 0.2 ms for recognition of a m.e.p.p. using a CBM 2001 microcomputer (Doherty *et al.*, 1986). In the latter case, individual values for peak amplitude and time from rise to half-fall were stored for subsequent analysis. The coefficient of variation (c.v.) of m.e.p.p. amplitude was determined for samples of >70 m.e.p.ps. Total m.e.p.p. frequencies were determined over 1 min or the time taken to collect 255 m.e.p.ps whichever was the shorter. The initial fall of frequency induced by crotoxin was measured as the time to half-fall, the time being interpolated from a least squares fit of the logarithm of the frequency (Caratsch *et al.*, 1985). Giant m.e.p.ps were defined as spontaneous potentials equal to or larger than 3 times the modal amplitude of m.e.p.ps recorded at the endplate. G.m.e.p.p. frequency (min^{-1}) was determined from a collection period, usually of 5 min but no shorter than 3 min.

The composition of normal Ringer solution was (mM): KCl 2.5, NaCl 111, $CaCl_2$ 1.8, HEPES buffer 5.0, pH 7.0. No adjustment of osmolarity was made in Ringer with a raised divalent cation concentration: sucrose experiments showed that these small osmotic changes did not affect the experimental outcome. Sulphate-Ringer solution was of the composition (mM): Na_2SO_4 93, K_2SO_4 1.0, $CaSO_4$ 1.8, tris(hydroxymethyl)aminomethane 4.0 (Molenaar *et al.*, 1987) adjusted to pH 7.0. Control muscles in sulphate-free solution were incubated in HEPES-Ringer as above but with KCl 2.0 mM. Isethionate-Ringer solution was made either by replacement of NaCl in HEPES-Ringer by Na isethionate to give a final chloride concentration of approximately 10 mM or by complete replacement of the remaining chloride salts by K_2SO_4, $CaSO_4$ and Tris buffer, respectively. As no difference was observed between the two solutions, the results were pooled.

The cutaneous-pectoris muscle was pinned, at no more than the *in situ* length, to the Sylgard resin in the base of the bath. Endplates were selected on the basis of large control m.e.p.p. amplitudes and, in the cutaneous-pectoris muscle, endplates near the medial edge were used. This procedure resulted in the sampling of a population of smaller diameter fibres (Banner & Hererra, 1986) exhibiting relatively low m.e.p.p. frequencies (Kuno *et al.*, 1971). In order to study the initial phase of the crotoxin response, basal m.e.p.p. frequency was raised by the addition of sucrose (final concentration 50 mM) to many of the Ringer solutions. (Doherty *et al.*, 1986). Control experiments showed that the response to the toxin was independent of sucrose. In some experiments tetrodotoxin (TTX, Koch-Light Laboratories, Colnbrook, England; final concentration 1 μM) was added to the Ringer to prevent any onset of twitching and, in a few experiments, neostigmine methylsulphate (3 μM final concentration) was also present. At each endplate, m.e.p.ps were recorded for a control period of about 10 min then, with the microelectrode *in situ*, the bathing solution (1.5 ml) was exchanged over 45 s by superfusion with 10 ml of medium containing crotoxin at a final concentration of 250 nM. The same procedure was used for washing with medium only. In experiments with AH5183, the nerve was stimulated by a suction electrode using rectangular pulses of 0.01 ms duration and 1–4 V intensity. AH5183 (2-[4-phenylpiperidino]-cyclohexanol hydrochloride) was a kind gift from Glaxo Group Research Ltd., Ware. Experiments were carried out at room temperature (18–22°C). Results are expressed as mean values ± s.e.mean and were analyzed for significance of difference using Student's *t* test (two-tailed).

Phospholipase A_2 activity

Phospholipase A_2 activity was measured with the use of dipalmitoyl (2-[1-^{14}C] palmitoyl) phosphatidylcholine (Amersham, England; CFA 601) as substrate in a thin-layer chromatographic (t.l.c.) assay. Phosphatidylcholine 400 nM (unlabelled soybean phosphatidylcholine and 20,000 c.p.m. labelled tracer) in a buffer containing 100 mM NaCl, 0.9 mM $CaCl_2$, 100 mM MOPS pH 7.0, was shaken at 22°C for 15 min with 0.15 nM crotoxin. The final reaction volume was 0.25 ml. $MnCl_2$ (90 mM) was added as appropriate to give final Mn ion concentrations indicated in Figure 4. The reaction tubes were put on ice, quenched with ice-cold 0.1 M EGTA (0.3 ml) and diluted with $CHCl_3$:CH_3OH:CH_3COOH (2:4:1) (0.2 ml). After adding 0.12 ml $CHCl_3$, the tubes were vortexed and centrifuged (2 min, 15,000 *g*). The upper (aqueous) phase was removed by aspiration and the lower phase was evaporated to dryness, dissolved in $CHCl_3$:CH_3OH (2:1) (0.05 ml) and chromato-

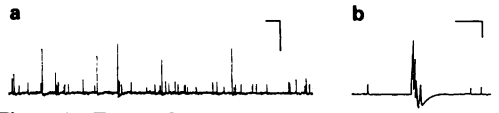

Figure 1 Types of spontaneous potentials recorded at two endplates after the addition of crotoxin (250 nM final concentration). (a) G.m.e.p.ps and m.e.p.ps recorded at an endplate 53 min after the addition of crotoxin in normal Ringer containing tetrodotoxin (TTX, 1 μM final concentration). (b) Large spontaneous potentials of the burst-type and m.e.p.ps recorded at an endplate 11 min after the addition of crotoxin in normal Ringer containing neostigmine methylsulphate (3 μM) and TTX (1 μM). Vertical scale: 1 mV; horizontal scale: 1 s. The undershoot is due to a.c. recording. Records retouched for clarity of reproduction.

graphed on silica gel t.l.c. plates as previously described (Strong *et al.*, 1976; Strong & Kelly, 1977).

Results

Early changes in m.e.p.p. frequency and m.e.p.p. amplitude distribution

Exposure of endplates to crotoxin 250 nM in Ringer solution (with or without 50 mM sucrose) induced a characteristic pattern of changes in m.e.p.p. frequency. The first phase was a rapid fall in mean m.e.p.p. frequency which subsequently reached a low steady state within 5 min. The second phase was characterized by a progressive rise in m.e.p.p. frequency, the onset of giant spontaneous potentials ($\geqslant$3 times modal amplitude) and occasional, more temporally dispersed large spontaneous potentials of the burst-type (Figures 1, 2). These changes were observed in the presence of TTX (1 μM final concentration). In contrast to the first phase, the time course of the second phase showed a marked variability both within and between endplates. At a number of endplates, a short burst of large potentials of the burst-type (Figure 1b) and g.m.e.p.ps heralded the onset of the second phase, but in the majority of endplates g.m.e.p.ps arose shortly after the onset of the rise in m.e.p.p. frequency (Figure 2). At 13 endplates, the time of onset of g.m.e.p.ps was 15 $\pm$ 2 min in comparison with a time of 11 $\pm$ 1 min for the onset of the second phase. The g.m.e.p.p. frequency remained low until about 30 min and thereafter rose at a variable rate. The amplitudes of g.m.e.p.ps were also variable with many of 3–8 $\times$ modal value (Figure 1a); their increase in number was reflected by a progressive rise in the coefficient of variation (c.v.) of m.e.p.p. amplitudes (Figure 2).

The second phase was associated with a steady rise in m.e.p.p. frequency and, at the time when the

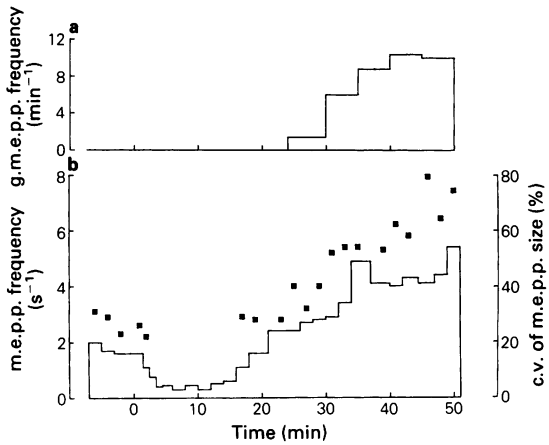

Figure 2 The time-course of changes in spontaneous transmitter release induced by crotoxin at an endplate in normal Ringer containing 50 mM sucrose. Crotoxin (250 nM final concentration) was added at 0 min and the preparation was washed with sucrose-Ringer at 32 min. (a) G.m.e.p.p. frequency: g.m.e.p.ps were defined as $\geqslant$3 $\times$ modal value and the average frequency in a 5 min collection period is plotted. (b) Total m.e.p.p. frequency and coefficient of variation (c.v.) of m.e.p.p. amplitudes (■). The collection period was either 1 min or 255 m.e.p.ps. The amplitude c.v. is plotted for m.e.p.p. samples of >70. Control mean m.e.p.p. amplitude was 0.35 mV. Temperature 20.8°C.

amplitude c.v. had risen from the control level of 26.3 $\pm$ 0.9% (n = 15) to >55%, the mean frequency had risen from 1.4 $\pm$ 0.4 s^{-1} to 2.6 $\pm$ 0.7 s^{-1} for endplates in Ca-Ringer solution. The effect was irreversible. Washing the preparation slowed the rate of rise of m.e.p.p. frequency and, at some endplates, even temporarily lowered it although the amplitude c.v. continued to rise. In general, no close parallel was observed between the rates of rise of total m.e.p.p. and g.m.e.p.p. frequencies. Bursts of m.e.p.ps were not a feature of intoxication and, in this study, short bursts (usually <1 s and no longer than 2 s) were observed infrequently during the early phase of rising m.e.p.p. frequency. Prolonged high frequency bursts were not recorded until the amplitude c.v. had risen above 55% and, with washing, these bursts could be delayed to 90 min or longer after first applying the toxin.

To determine if the characteristics of the second phase of intoxication could be mimicked by a small generalized increase in Ca influx, endplates were exposed to Ringer containing Ca 6.3 mM and K 5–10 mM (with or without sucrose) to raise the basal m.e.p.p. frequency to comparable levels. After more than 30 min exposure, there were no g.m.e.p.ps and the amplitude c.v. was unchanged (29.0 $\pm$ 1.3% at a

Table 1 The effect of modified Ringer solution containing various concentrations of $CaCl_2$ and of $MnCl_2$ on the biphasic changes in m.e.p.p. frequency induced by crotoxin (250 nM final concentration)

Cation concentration Ca (mM)	Mn (mM)	*Initial phase: time to half-fall* (min) (n)	*Secondary phase: time to onset of rise* (min) (n)
0.9	0	2.2 ± 0.3 (6)	10.8 ± 0.9 (7)
1.8	0	2.6 ± 0.2 (7)	11.1 ± 1.3 (13)
4.5	0	2.5 ± 0.6 (3)	12.1 ± 1.6 (3)
6.3	0	3.0 ± 0.4 (4)	14.3 ± 1.7 (3)
0.9	1.8	2.9 ± 0.2 (3)	13.3 ± 1.6 (3)
0.9	3.6	7.2 ± 0.8*** (6)	25.6† ± 4.2** (3)
0.9	5.4	18.7 ± 5.3* (4)	48.5 ± 5.6*** (4)

Results are means ± s.e.mean with n the number of preparations. † Onset time > 37 min at 2 other endplates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus values at 0.9 mM Ca.

m.e.p.p. frequency of $3.1 \pm 0.6\,s^{-1}$, 10 endplates, 2 muscles). These results were unaffected by prolonging the exposure to 60 min or by raising the frequency to $16\,s^{-1}$ with K 15 mM/Ca 6.3 mM-Ringer.

Lowering the temperature to 12 ± 1°C from 19 ± 1°C delayed the onset of the second phase

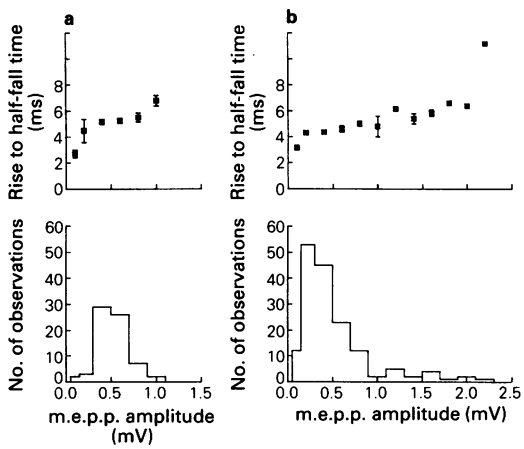

Figure 3 Time from rise to half-fall (upper graphs) and frequency distribution (lower histograms) plotted as a function of m.e.p.p. amplitude: (a) before the addition of crotoxin (250 nM final concentration), (b) at 52 min after the addition of crotoxin. At (a), the mean m.e.p.p. frequency was $0.2\,s^{-1}$ and the collection period was 5 min; at (b), the mean frequency was $2.5\,s^{-1}$ and the collection period was 1 min. The data are grouped in amplitude bins of 0.2 mV except for the first bin of 0.1 mV (threshold 0.1 mV). The time to half-fall is given as mean ± s.e.mean and is uncorrected for errors due to the finite trigger threshold. Estimated errors are 1, 0.5 and 0.3 ms for bins 1, 2 and 3 respectively. Records obtained at an endplate (same as Figure 1a) in normal Ringer containing tetrodotoxin (1 μM final concentration). Coefficient of variation of amplitude distribution (a) 29%, (b) 69%.

(≥34 min, n = 3 compared to 12 ± 1 min, $n = 8$). At the lower temperature, the basal m.e.p.p. frequency was too low to determine the initial phase of action of crotoxin.

The effect of divalent cations Neither the time taken for the m.e.p.p. frequency to fall by one half ($t_{50\%}$) nor the onset of the second phase, was affected by external Ca concentrations over the range 0.9–6.3 mM (Table 1). The rise in g.m.e.p.p. frequency was also unaffected and amplitude c.v. >55% values were recorded at endplates in 0.9 and 6.3 mM Ca. Furthermore, once the second phase had started, it could not be reversed by removing external Ca ions. M.e.p.p. amplitude c.v. rose to 90.2 ± 4.1% (5 endplates, 3 muscles) after exchanging Ca 0.9 mM-sucrose-Ringer solution for sucrose-Ringer lacking added Ca ions.

Crotoxin was ineffective when added to a Ca-free medium containing Sr 4 mM-EGTA 1 mM. No characteristic changes in m.e.p.p. frequency were observed over 75 min at 3 endplates. Whilst at a fourth endplate a rising phase was observed after 50 min. However no rise in g.m.e.p.p. frequency was observed at any endplate.

Mn ions (1.8 mM) in Ca 0.9 mM-Ringer affected neither the primary nor secondary phases of intoxication. However, higher concentrations of Mn (3.6 and 5.4 mM) in Ca 0.9 mM-Ringer significantly slowed the initial rate of fall of m.e.p.p. frequency and delayed the onset of the second phase (Table 1). Once this phase was established, m.e.p.p. amplitude distributions with a c.v. >55% were recorded in all Ca-Mn solutions, including Ca 0.9 mM-Mn 5.4 mM solution (5 endplates, 4 muscles). Control experiments showed that the lowest Mn Ringer solution (Ca 0.9 mM-Mn 1.8 mM) blocked all neurally evoked e.p.p.s.

Time-course of g.m.e.p.ps The time from rise to half fall of m.e.p.p. amplitude, plotted as a function of

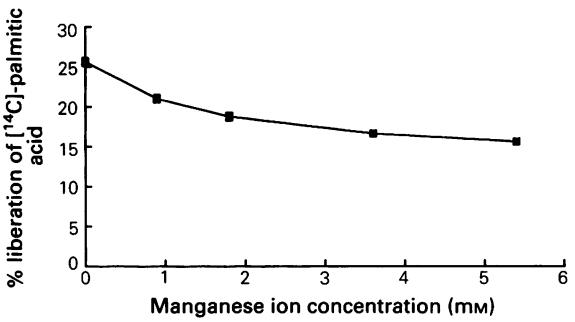

Figure 4 Phospholipase A_2 activity of crotoxin (250 nM final concentration) measured in Ca 0.9 mM-Ringer at varying concentrations of $MnCl_2$. Liberation of [^{14}Cl-palmitic acid from [^{14}C]-phosphatidylcholine was determined after 15 min incubation at 22°C (see Methods). Each point is the mean value of 4 determinations (s.e. mean bars fall within the symbol).

m.e.p.p. amplitude, showed a small but progressive rise in value with increasing amplitude. This trend was observed at all endplates, both before and after the addition of 250 nM crotoxin and at a time when the onset of g.m.e.p.ps ($\geq 3\times$ modal) was established (Figure 3). In 7 preparations, bathed in modified Ringer containing various concentrations of Ca and Ca/Mn ions, measurements were made 30–90 min after exposure to crotoxin, and at a time when the frequency of g.m.e.p.ps was $21.0 \pm 4.5\,min^{-1}$. The mean time from rise to half fall of these giants was $145.7 \pm 4.5\%$ of the mean value of the modal population; this is no more than the variation seen in control populations of time from rise to half fall according to m.e.p.p. amplitude. Thus the time-course of g.m.e.p.ps is similar to that of the m.e.p.p. population.

The effect of Mn ions on the phospholipase A_2 activity of crotoxin

In common with all other phospholipase A_2 enzymes, Ca ions are an essential co-factor for the full expression of crotoxin's enzymatic activity (Breithaupt, 1976). With artificial dipalmitoyl phosphatidylcholine phospholipid membranes as substrate, the added presence of increasing concentrations of Mn ions partially inhibited the enzyme activity of crotoxin in a dose-dependent manner (Figure 4). Inhibition increased with increasing Mn concentrations up to 5.4 mM $MnCl_2$, but less than 50% of the enzymatic activity of crotoxin was inhibitable by a 50 fold molar excess of Mn over Ca ions. Unlike other metal ion inhibitors of phospholipases A_2 (e.g. Sr, Ba), inhibition by Mn for the Ca ion binding site on crotoxin was not competitive and further analysis failed to characterize any simple inhibitory mechanism. Mn ions completely failed to support enzyme activity of crotoxin in the absence of Ca ions.

Induction of g.m.e.p.ps

The effect of Ringer lacking chloride ions As the onset of g.m.e.p.ps during recovery from high K stimulation has been linked to a chloride-dependent process (Molenaar *et al.*, 1987), we were interested to determine whether crotoxin-induced g.m.e.p.ps shared this property. Muscles were bathed in sulphate-Ringer (Molenaar *et al.*, 1987) containing sucrose and TTX to prevent twitching. When endplates were sampled from 30–100 min after the addition of crotoxin, the frequency of g.m.e.p.ps was significantly ($P < 0.001$) reduced in sulphate-Ringer ($1.4 \pm 0.3\,min^{-1}$, 20 endplates in 4 muscles) in comparison with values in chloride-Ringer ($5.7 \pm 0.8\,min^{-1}$, 25 endplates, in 4 muscles including 3 contralateral muscles). Prior to the addition of the toxin, mean g.m.e.p.p. frequency was usually $<0.2\,min^{-1}$ and no more than $0.4\,min^{-1}$ at any endplate. Crotoxin in sulphate-Ringer also failed to induce an initial fall in m.e.p.p. frequency; at 10 min after the addition of crotoxin, mean m.e.p.p. frequency was still $103 \pm 3.5\%$ of pre-toxin ($n = 20$) in sulphate-Ringer in comparison with a crotoxin-induced fall to $24 \pm 1\%$ of pre-toxin value ($n = 25$) in chloride-Ringer. Furthermore, the depression in g.m.e.p.p. frequency was associated with a significant ($P < 0.01$) reduction in total m.e.p.p. frequency at endplates in sulphate-Ringer ($1.5 \pm 0.4\,s^{-1}$, $n = 20$) in comparison with values obtained at endplates in chloride-Ringer ($3.3 \pm 0.4\,s^{-1}$, $n = 25$). It proved not possible to determine reproducibly the phospholipase A activity of crotoxin in sulphate-Ringer. This suggests that sulphate anions may interfere with a number of processes, possibly due to their ability to adsorb to lipid bilayers and proteins as well as to reduce markedly the free-calcium ion concentration (see review by Bretag, 1987).

As isethionate anions are widely used as a substitute for chloride ions (Bretag, 1987), we decided to re-investigate the effect of these anions on spontaneous transmitter release. In control endplates in isethionate-Ringer, m.e.p.p. amplitude distributions were normal over 120 min of recording (amplitude) c.v. of $25.7 \pm 0.9\%$, 15 endplates, 2 muscles). This is in marked contrast to the high reversible incidence of g.m.e.p.ps ($>2\times$ modal) reported by Ashford & Wann (1983). The two studies used different procedures for mounting the preparations and it is possible that isethionate anions predispose the terminal for such a response. The addition of crotoxin to isethionate-Ringer induced the characteristic fall and

secondary rise in m.e.p.p. frequency. When sampling endplates from 30–100 min after exposure to crotoxin, the frequency of g.m.e.p.ps in isethionate-Ringer was $4.3 \pm 0.6\,min^{-1}$ (9 endplates, 2 muscles) in comparison with frequency of $5.0 \pm 1.5\,min^{-1}$ at endplates in chloride-Ringer (10 endplates in 2 contralateral muscles). No chloride-dependent process was apparent in the induction of g.m.e.p.ps by crotoxin.

The effect of AH5183 (vesamicol) We have investigated the possibility that g.m.e.p.ps induced by crotoxin arise from changes in vesicle formation by determining the effect of crotoxin in Ringer containing AH5183 (10 μM final concentration), an inhibitor of vesicular acetylcholine transport at the frog neuromuscular junction (Van der Kloot, 1986). To optimize conditions for the inhibition of acetylcholine transport into newly formed synaptic vesicles in the presence of crotoxin, the following protocol was used: each preparation (with or without AH5183) was stimulated at 0.4 Hz for 5 min to allow time for diffusion and then at 4 Hz for 6 min to ensure vesicle recycling. At least 15 min later, when m.e.p.p. frequencies were stable (mean of $1.7\,s^{-1}$), crotoxin and TTX were added to the bath. In each of 4 paired muscles, crotoxin induced a rapid fall in m.e.p.p. frequency followed by a progressive rise; g.m.e.p.ps appeared either shortly before or after the onset of the secondary phase. However, when the frequency of g.m.e.p.ps was measured at endplates sampled between 35 min–100 min after the addition of crotoxin and TTX, a significantly reduced incidence of $1.7 \pm 0.1\,min^{-1}$ (23 endplates) was observed in the presence of AH5183 in comparison with a value of $3.1 \pm 0.3\,min^{-1}$ (21 endplates, $P < 0.001$) in control preparations. The m.e.p.p. frequencies at these endplates were similar ($6.1 \pm 0.6\,s^{-1}$ in the presence of AH5183 and $5.8 \pm 0.8\,s^{-1}$ in its absence).

Discussion

Phospholipase A_2 activity

Crotoxin induced, without appreciable delay, a marked and rapid fall in the rate of spontaneous transmitter release which was followed by a slow secondary rise in the rate of both m.e.p.p. and g.m.e.p.p. frequencies. Both phases were inhibited in Ca-free Ringer containing Sr ions, an artificial condition which fully maintains transmitter release (Dodge *et al.*, 1969), and in which the phospholipolytic activity of crotoxin is very low (Chang *et al.*, 1977). High concentrations of Mn (in 0.9 mM Ca-Ringer) slowed both crotoxin-induced phases, an effect not due to electrostatic screening as similar calcium concentrations did not alter the timing of these phases. However, these Mn-Ca Ringer solutions partially inhibited the enzymatic activity of crotoxin on an artificial substrate. The above findings suggest that the phospholipase A_2 moiety of crotoxin is involved in both the initial decline and secondary rise in m.e.p.p. frequency. This is consistent with a binding of the basic phospholipase A_2 subunit to the target site and subsequent dissociation of the acidic subunit (Bon *et al.*, 1979).

Characteristics of g.m.e.p.ps

The occurrence of an altered m.e.p.p. amplitude distribution at neuromuscular junctions is characteristic of intoxication by phospholipase A_2 neurotoxins, both *in vivo* and *in vitro*. In the present study, the similarity of the timecourse of g.m.e.p.ps to that of m.e.p.ps at the same junction, indicates that g.m.e.p.ps are the result of an increased transmitter release from the motor nerve terminals. However, crotoxin-induced g.m.e.p.ps are unlikely to arise from the synchronous activation of a number of release sites, as might occur from localized excitation of the nerve terminal, since g.m.e.p.ps were observed in the presence of TTX-blockade of voltage-dependent Na channels (see also Hawgood & Santana de Sa, 1979) as well as Mn-blockade (Baker & Glitsch, 1975) of voltage-dependent Ca-channels. These g.m.e.p.ps also persisted in Ringer to which no Ca had been added. At control endplates, depolarization of motor nerve terminals by a raised external K concentration failed to induce g.m.e.p.ps (Ashford & Wann, 1983; Molenaar *et al.*, 1987), even in the presence of a raised external Ca concentration as in the present study.

Crotoxin-induced g.m.e.p.ps were not associated with any prior period of intensive release of transmitter as bursts of m.e.p.ps were both of very short duration and low incidence; substantial numbers of g.m.e.p.ps were present at a mean quantum release of $2.6\,s^{-1}$. This is in contrast to β-bungarotoxin poisoning where the secondary phase is characterized by high frequency bursts of m.e.p.ps (Abe *et al.*, 1976; Caratsch *et al.*, 1985).

It is of interest that g.m.e.p.ps that arose during recovery from a massive discharge of transmitter have been attributed to alterations in membrane retrieval (Heuser, 1974; Miller & Heuser, 1984). Such g.m.e.p.ps have a number of characteristics (Molenaar *et al.*, 1987) shared by crotoxin-induced g.m.e.p.ps, although a chloride-dependent link was not apparent in the present study. Evidence that suggests that crotoxin induces a disturbance in vesicle formation was obtained by use of AH5183 (vesamicol), an inhibitor of acetylcholine transport into purified synaptic vesicles (Anderson *et al.*, 1983) as well as into vesicles within the motor nerve ter-

minal (Van der Kloot, 1986; Whitton *et al.*, 1986). AH5183 reduced the incidence of g.m.e.p.ps without affecting either the time course and magnitude of crotoxin-induced alterations in m.e.p.p. frequency or the time of onset of g.m.e.p.ps. Electron microscopic studies of intoxicated motor nerve terminals also indicate that phospholipase A_2 neurotoxins, including crotoxin, may alter the characteristics of membrane retrieval as omega-shaped pits (Heuser & Reese, 1973) characteristically increased in number at an early stage of intoxication when synaptic vesicles remained unchanged in number and appearance (Chen & Lee, 1970; Cull-Candy *et al.*, 1976; Strong *et al.*, 1977; Gopalakrishnakone & Hawgood, 1984). The delayed but progressive increase in distribution of m.e.p.p. amplitudes observed after the addition of crotoxin may be due, at least in part, to prior changes in vesicle formation.

We suggest that the phospholipase A_2 subunit of crotoxin induces changes in areas of the axolemmal membrane involved in exocytosis and endocytotic membrane retrieval, possibly by localized alterations in membrane fluidity, thus leading to the characteristic changes in m.e.p.p. frequency and amplitude distribution. One of the presynaptic phospholipase neurotoxins is known to hydrolyze preferentially artificial phospholipid membranes undergoing phase transitions (Strong & Kelly, 1977). As both exocytosis and endocytotic membrane retrieval intrinsically produce transient phase boundaries, such events may facilitate access of the phospholipase A_2 moiety of these neurotoxins to their substrate within the phospholipid bilayer. We suggest that the active zone is a prime target for these frequency-dependent neurotoxins (Strong, 1987).

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